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Overwintering temperatures affect freezing temperatures of turions of aquatic plants

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ABSTRACT

The effect of different overwintering temperatures (2.5 ± 1 °C in a refrigerator or outdoor natural overwintering on wet topsoil with weak frosts) on the freezing temperature and survival rate of turions of 10 aquatic plant species with different ecological traits (free-floating habit or bottom rooting) was studied using mini thermocouples. Dormant, non-hardened turions of 9 species exhibited freezing within a narrow temperature range of −7.0 to −10.2 °C, while *Hydrocharis morsus-ranae* froze at −3.6 °C. The survival rate of the turions after the measurements was, however, very low (0–38%). In several species, the freezing temperature of turions at the beginning of germination was not significantly different (at $p < 0.05$) from the dormant ones. The mean freezing temperature of outdoor hardened turions of 6 species was within a very narrow range of −2.8 to −3.3 °C and was thus significantly higher by 4–7 °C ($p < 0.0002$) than that for the non-hardened turions. It is assumed that the freezing temperatures indicate freezing of the extracellular water. The hardened turions of all 7 species were able to survive mild winter frosts under the topsoil conditions at a rate of 76–100%. These characteristics suggest that the turions of aquatic species can be hardened by weak frosts and that their frost hardiness is based on the shift from frost avoidance in non-hardened turions to frost tolerance.

Introduction

Turions (winter buds) are vegetative dormant organs produced by perennial aquatic plants in response to unfavorable ecological conditions (Sculthorpe, 1967; Bartley and Spence, 1987) and are formed by extreme condensation of short, modified leaves in the shoot apex. These tough, sturdy organs form at the end of the growing season and can be spherical (*Utricularia*), rhomboid (*Aldrovanda*) or greatly enlarged or flat (*Potamogeton* spp., *Caldesia*). As overwintering organs, turions are partly frost resistant while their fragile mother shoots are not (Winston and Gorham, 1979a; Adamec, 1999a). Turions of all aquatic plant species usually escape from being included in ice. They overwinter and break their innate dormancy at the bottom of an aquatic habitat in darkness, under hypoxic or anoxic conditions, usually lightly covered by organic sediments. Turions of several species can also be drought resistant (Maier, 1973a,b; Adamec, 2008a). They are also storage organs and, in autumn, they accumulate starch (9–70% dry weight, DW) and free sugars (in total 7–14% DW; Winston and Gorham, 1979a; Ley et al., 1997; Adamec, 1999b, 2003; Weber and Noodén, 2005). As is typical of storage organs, the dark respiration rate at a standard temperature of turions of aquatic species is rather low compared to dark respiration rates of shoots (leaves) of aquatic plants of the same or other species (Adamec, 2008a, 2003, 2011). The turions of aquatic plants also act as storage organs for mineral nutrients (N, P), although this storage function (*sensu* Chapin et al., 1990) is presumably less distinct than that for carbohydrates (Adamec, 2010).

Two distinct ecological strategies for their germination and sprouting may be distinguished (Sculthorpe, 1967; Bartley and Spence, 1987; Adamec, 2008b). Turions of free-floating aquatic genera (e.g., *Aldrovanda*, *Utricularia*, *Hydrocharis*) ripen at the water surface, overwinter at the bottom, then usually germinate and sprout at the water surface when the water warms. Turions of aquatic rooted genera, however, (e.g., *Caldesia*, *Myriophyllum*, *Potamogeton*) ripen, germinate, sprout and root at the bottom in colder water and in shade. Moreover, turions of free-floating aquatic species differ in their mechanisms of autumnal sinking and spring floating (Newton et al., 1978; Adamec, 1999a,b, 2008b): Turions of *Aldrovanda vesiculosa*, *Hydrocharis morsus-ranae*, and *Spirodela polyrhiza* have developed an active mechanism of sinking and rising. It has been suggested that the sinking and rising of *Aldrovanda* turions is caused by variable gas volume in the gas spaces of turion leaves (Adamec, 1999b, 2003). On the contrary, *Utricularia* turions are less dense than water and are dragged by the decaying mother shoots to the bottom. By early spring, these turions separate and float to the surface (Adamec, 1999b). Two dormancy states and their hormonal patterns were described in detail for turions of Canadian *Utricularia*

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macrorhiza using bioassays (Winston and Gorham, 1979b). Turions entered a state of innate dormancy at the end of the summer, when their growth was blocked by turion endogenous factors. At the end of October, the turions entered a state of imposed dormancy when their germination depended only on higher temperature. The next stage in turion germination leads to the sprouting of new shoots and this occurs under sufficiently high temperature and light conditions. These two dormancy states were also described in *Aldrovanda vesiculosa* turions (Adamec, 2003). Resulting from these ecophysiological characteristics, turions of aquatic plants can also overwinter above the water surface on wet substrate and possess certain frost resistance. But the range and mechanisms (components) of this latter have never been studied (Adamec, 1999a, 2008a).

Plant tissues have two main strategies to cope with frost. They can either avoid freezing, for instance by transient supercooling, or they tolerate freezing by the formation of ice crystals in extracellular spaces, which prevents ice occurrence and subsequent damage in the living cells (Levitt, 1980; Sakai and Larcher, 1987; Larcher, 1995). These freezing-tolerant plants have the ability to survive long winters with severe frosts, provided that they have been properly acclimated at their dormant state, which involves a complex of biochemical and physiological processes. In herbaceous plants, winter frost hardiness is rapidly induced during autumn due to the regular occurrence of near-freezing temperatures and is lost during spring. Avoidance of freezing in general does not require entering a dormant state and the cells remain hydrated and metabolically active. Except for the special case of deep supercooling (e.g., typical for buds and some seeds), the disadvantage of this avoidance is that it provides only short-term protection against even relatively mild freezing temperatures (Levitt, 1980; Sakai and Larcher, 1987; Beck, 1994; Guy, 2003; Körner, 2003).

In this comparative study, using mini-thermocouples we studied the effect of different overwintering temperatures (temperature of $2.5 \pm 1\,^{\circ}C$ in a refrigerator or outdoor natural overwintering on a wet topsoil) on the freezing temperatures of turions of 10 aquatic plant species with different ecological traits (free-floating or bottom rooting) with regard to turion survival. Moreover, we also compared the freezing temperatures in germinating turions of four of these species. Thus, the aim was to elucidate the strategy of frost resistance of these turions and to prove that a certain frost resistance is induced by turion hardening under weak frosts.

Materials and methods

Plant material

Turions of the following aquatic plant species were used: *Aldrovanda vesiculosa* L. (Droseraceae; collected originally from E Poland), *Utricularia australis* R.Br. (Lentibulariaceae; from the Czech Republic), *U. bremii* Heer *ex* Kölliker (from NW Russia), *U. vulgaris* L., *U. ochroleuca* Hartm. s. str., *U. stygia* Thor (syn. *U. ochroleuca* Hartm. s. l.), *U. intermedia* Hayne (all the four latter species from the Czech Republic). These were grown outdoors in three plastic containers that simulated their natural conditions. A subtropical turion forming accession of *U. stellaris* Linn.f. (from NE N.S.W., Australia) was grown outdoors in a 15 l aquarium standing in cooling water in a larger plastic container. This subtropical accession was selected for a comparison with temperate species. The plants in all containers were grown in tap water with a litter of robust *Carex* species used as a substrate (for all details, see Sirová et al., 2003; Adamec, 2010). *Caldesia parnassifolia* L. (Alismataceae; from W Germany) was grown in a very shallow culture (3–5 cm) in a 300 l plastic container in a peaty substrate. Ripe turions of *Hydrocharis morsus-ranae* L. (Hydrocharitaceae) were collected from a natural site near Třeboň, S Bohemia, Czech Republic, in mid-October 2011 and 2012. The species selected for this study differ greatly in their functional traits: *Aldrovanda* and all *Utricularia* species are rootless, mostly submerged, carnivorous plants; *Hydrocharis* is a free-floating plant with roots and *Caldesia* is a typical rooted amphibious species. Except for *Caldesia*, all species usually germinate and sprout at the water surface, while *Caldesia* germinates, sprouts and roots at the bottom. After collection from the cultures between the end of September and mid-November, ripe turions of all species were washed using tap water and stored in the dark in the filtered cultivation medium in a refrigerator at $2.5 \pm 1\,^{\circ}C$ until used.

Freezing temperature measurement

Two sets of freezing temperature measurements were conducted using mini-thermocouples on different batches of turions at the state of imposed dormancy for two seasons. From the 2nd to the 13th March 2012, the measurements were conducted on turions of all 10 species that had overwintered in a refrigerator at $2.5 \pm 1\,^{\circ}C$. In *Aldrovanda*, *U. australis*, *U. bremii* and *U. ochroleuca*, the measurements were also made on turions which had been germinated in a growth chamber at $20 \pm 1\,^{\circ}C$ with a 12 h photoperiod for 1–6 d before the measurements. Turions of the former three species, which germinated for 2–5 d, bore distinct symptoms of germination (erected leaflets). These turions were never subject to frost and their overwintering mimics the natural, underwater conditions at the bottom of water bodies. The second set of measurements was conducted during the 7th–8th March 2013 on the turions of 6 species that had overwintered on a wet topsoil in an empty cultivation container outdoors (see below). This type of overwintering mimics the natural situation when autumnal turions are entangled in the wet organic substrate above the water surface and are thus subject to winter frosts (Adamec, 1999a). This can induce turion frost hardiness.

Freezing temperature measurements were made in a polystyrene box with an inner volume of 1.7 l, which was put into the freezer section of a commercial refrigerator. A temperature of −20 to −22 °C was maintained in the freezer. In order to decrease and optimize the cooling rate of the air inside the polystyrene box, plastic vials with saturated NaCl solution were placed in the box as a 'temperature buffer'. A few drops of distilled water were added to the box to moisten the air and a small fan homogenized the inner temperature inside the box. After putting a box into the freezer, the inner air temperature declined in an approximate negative exponential way and the cooling rate within the potential freezing temperature of −3 and −10 °C ranged between 2 and $5\,^{\circ}C\,h^{-1}$ (cf. Sklenář et al., 2010). The cooling rate corresponded exactly to that which was monitored on the wet organic topsoil (without snow cover) in an emptied cultivation container kept outdoors on evenings during the coldest days of the year (on 1–4 Feb. 2012; ca. $1.5\text{–}4\,^{\circ}C\,h^{-1}$). A set of 8 fine wire Cu–Co mini thermocouples (tip diam. 0.4–0.5 mm; EMS Brno, Czech Republic) for the estimation of the turion freezing temperature was placed in the polystyrene box and fixed by sealing tape. Stored turions were quickly washed with tap water and thoroughly blotted dry. In *Hydrocharis* and *Caldesia*, the outermost pale membraneous turion leaves were removed. Three turions of each species were blotted dry and weighed for fresh weight (FW) and, after the experiments, dry weight (DW; drying at 80 °C). At a room temperature of ca. 20 °C, the tips of the mini-thermocouples were inserted into the turions (between the leaflets) so that the turions were freely surrounded by air and the room temperature manipulation of turions never exceeded 10 min. The box was then closed and put into the freezer for the measurement, with 7 or 8 turions of each species of a similar size monitored simultaneously. Turion temperature was recorded by a 12-channel EdgeBox V12 datalogger (EMS Brno) every 6 s.

The measurement noise within one mini-thermocouple did not exceed ca. 0.15 °C and the difference between different sensors was ca. 0.2 °C. The recordings started from ca. 15–20 °C and ended at ca. −8 to −11 °C, after an exotherm peak, a sudden transient increase in the turion temperature of around 2–5.5 °C of a duration of 1–10 min (depending on turion size) indicated that the water inside all the turions had been frozen (Sklenář et al., 2010). The lowest temperature immediately preceding this temperature increase was estimated from the recordings as the freezing temperature (exotherm temperature). After measurement, the turions of some species were transferred to a growth chamber and allowed to germinate in the filtered cultivation medium at 20 ± 1 °C with a 12 h photoperiod, to establish if they have survived. Control turions of all species, which overwintered in the refrigerator under the same conditions, were able to germinate at 98–100% ($n = 30$–40).

After the freezing temperature measurement, 8 dead turions of *U. ochroleuca*, which were found in the growth chamber after four days, were used in another measurement to estimate the freezing temperature of dead turions. *U. australis* turions, which had been germinated for 1 d, were allowed to thaw at room temperature for 10 min after the first measurement, being continuously attached to the mini-thermocouples, and were measured again. At the end of the first measurement, the temperature in the box dropped to ca. −11 °C. It is thus probable that the turions were already dead after the first measurement. To compare freezing temperatures in turions and non-dormant, growing shoot apices, such measurements were also conducted on 7–9 mm long shoot apices of a tropical accession of *A. vesiculosa* (collected from Darwin, N.T., Australia) which was grown in an indoor aquarium.

Winter hardening

To confirm that autumnal turions at the state of innate dormancy are able for frost-hardening, turions of 7 species (collected between 3 October and 18 November 2012) were taken from the refrigerator and allowed to overwinter under nearly-natural conditions on a wet substrate outdoors. In this way they were exposed to natural winter frosts that might induce frost hardiness. On 20th November 2012, 60 turions of each of these species of homogeneous size were randomly selected and subdivided into 5 groups of 12 turions each. Twelve turions of each of the 7 species were put into 5 porous (mesh size 1.5 mm) polyester bags 11 cm × 9 cm and placed on wet topsoil in an emptied cultivation container. The container was used for seasonal growing of *U. vulgaris*, *U. stygia* and *Aldrovanda* and ca. 1 cm layer of partly decomposed *Carex* litter with detritus overtopped a 6 cm layer of sand. The bags with turions were gently flattened onto the topsoil so that the turions were in contact with the wet substrate and could not dry out. Two Minikin T (EMS Brno) temperature dataloggers were placed between the bags and were one-third immersed in the litter; they measured the top soil temperature every 30 min. To further prevent the turions in the bags from drying out, the bags and dataloggers were covered by a sheet of a white filter paper (40 cm × 36 cm) which was fixed to the topsoil by plastic bands. On 7th March 2013, turions were removed from four bags and allowed to germinate in filtered cultivation medium in the growth chamber at 20 ± 1 °C with a 12 h photoperiod (all turions from each bag separately; $n = 4$). The first signs of turion sprouting (Adamec, 2011) were considered as survival. Eight turions of each species from the fifth bag were thoroughly washed, blotted dry, and used for the freezing temperature measurements as above. Again, 3–4 turions were weighed for FW and DW. The turions were exposed to −8 to −10 °C during the measurements and their survival was tested in the growth chamber. The measurement was also conducted on *U. stygia* turions, which overwintered on the wet topsoil uncovered by the filter paper.

The mean topsoil temperature was 0.4 ± 2.0 (SD) °C over the whole exposure winter period of 108 days. The maximum temperature was ca. 7.0 °C as early as on 25 November. In total, 75 days with continuous frost were recorded but the frosts were very mild (mean −0.6 ± 0.5 °C) with only 13 days when the topsoil temperature shortly dropped below −2.0 °C. The lowest temperature (ca. −4.2 °C) was measured during the first ten days of February, when snow cover disappeared; this snow cover otherwise effectively protected the topsoil from deep frosts. It has been shown that the dataloggers covered by the filter paper recorded temperatures 1–1.2 °C lower than uncovered ones.

Statistical treatment

Throughout this paper, the mean with standard error is shown wherever possible; $n = 7$–8 turions for freezing temperatures; $n = 4$ blocks each with 12 turions for assessment of survival of hardened turions. Significant differences in freezing temperatures between differently germinated variants within one species were evaluated by a one-way ANOVA (Tukey HSD test). The same test was also used for looking for the significant differences in freezing temperatures between non-hardened and hardened turions within each species.

Results

Dormant, non-hardened turions of 7 *Utricularia* species, *Aldrovanda* and *Caldesia* exhibited freezing within a narrow temperature range of −7.0 to −10.2 °C, while *Hydrocharis* froze at −3.6 °C (Table 1). The turions of subtropical *U. stellaris* froze at the same temperature as other temperate *Utricularia* species. In *Aldrovanda*, *U. australis*, *U. bremii* and *U. ochroleuca*, the freezing temperatures of turions at the state of breaking imposed dormancy or even bearing distinct symptoms of germination were not significantly different, at $p < 0.05$, from the dormant ones. Moreover, the freezing temperature of dead *U. ochroleuca* turions did not significantly differ from that of living dormant turions. After *U. australis* turions (germinated for 1 d) had been allowed to thaw shortly after the first measurement, their freezing temperature during the repeated measurement (−9.2 ± 0.6 °C; data not shown) did not differ significantly from that at the first measurement. However, the freezing temperature of growing shoot apices of tropical *Aldrovanda* was significantly lower (−8.5 ± 0.2 °C, $p < 0.00034$; data not shown) than that of dormant *Aldrovanda* turions (−7.0 ± 0.2 °C). Yet the DW proportion in the shoot apices was only 10.8% of FW. On the other hand, the DW proportion in dormant, non-hardened turions of all species was 20.4–30.5% FW (Table 1). No non-hardened turions survived the freezing temperature measurements (exposure to ca. −8 to −11 °C) in *Aldrovanda*, *U. australis*, *U. ochroleuca* and *U. stygia*, but 37.5% of dormant turions of *Caldesia* and 12.5% of 2-d germinated *U. bremii* turions survived and sprouted.

The mean freezing temperature of outdoor hardened turions of all 6 species measured was within a very narrow range of −2.8 to −3.3 °C (Table 2). Within each species, the value for the hardened turions was significantly higher by 4–7 °C ($p < 0.0002$) than that for the non-hardened turions overwintered in a refrigerator at 2.5 ± 1 °C. The value for the *U. stygia* turions overwintered below the sheet of the filter paper was exactly the same as that for those turions overwintered next to the paper. When compared to the turions overwintered in a refrigerator (Table 1), the DW proportion of those overwintered outdoors was some 1.7–16 (median 6.5) percentage points greater. Generally, the hardened turions of all 7 species were able to survive mild winter frosts under topsoil conditions at 76–100% and also the turion survival after the

Table 1
Freezing temperatures of turions of aquatic plants after overwintering in a refrigerator at 2.5 ± 1 °C, simulating natural conditions under water at the bottom of lakes or rivers.

Species	Preconditioning treatment	DW (% FW)	Freezing temp. (°C)	Survival after measur. (%)
Aldrovanda vesiculosa	–	23.3	−6.95 ± 0.23[a]	0
-"-	20 °C for 2 d	20.2	−6.87 ± 0.22[a]	–
-"-	20 °C for 4 d*	17.4	−6.68 ± 0.19[a]	–
Utricularia australis	–	30.5	−8.98 ± 0.34[a]	0
-"-	20 °C for 1 d	34.4	−8.32 ± 0.49[a]	–
-"-	20 °C for 2 d*	24.9	−8.12 ± 0.46[a]	–
Utricularia bremii	–	27.2	−7.90 ± 0.40[a]	–
-"-	20 °C for 2 d	29.1	−8.45 ± 0.49[a]	12.5
-"-	20 °C for 5 d*	25.8	−7.82 ± 0.60[a]	
Utricularia ochroleuca	–	26.5	−9.36 ± 0.18[a]	0
-"-	20 °C for 3 d	27.1	−8.59 ± 0.35[a]	–
-"-	20 °C for 6 d	23.3	−9.24 ± 0.24[a]	–
-"-	Dead turions after freezing measurement	–	−8.73 ± 0.22[a]	
Utricularia vulgaris	–	20.4	−7.12 ± 0.36	–
Utricularia stellaris	–	24.0	−8.56 ± 0.23	–
Utricularia intermedia	–	27.8	−10.23 ± 0.28	–
Utricularia stygia	–	23.8	−10.05 ± 0.39	0
Caldesia parnassifolia	–	29.7	−8.61 ± 0.22	37.5
Hydrocharis m.-ranae	–	22.0	−3.59 ± 0.09	–

In some cases, turions were kept before the measurement at 20 °C ("preconditioning treatment") to germinate for 1–6 days and those labeled by an asterisk started germinating. Dry weight (DW) expressed in % of fresh weight (FW). The same letter within each species denotes a statistically non-significant difference at $p > 0.05$ (one-way ANOVA, Tukey HSD test). Means ± SE are shown; $n = 8$. –: not measured.

measurements (exposure up to −8 to −10 °C) was much higher (usually 75–100%) than in the case of non-hardened turions (Table 2). Thus, outdoor turion hardening led to a marked increase of the freezing temperature but, simultaneously, to an increase of their frost resistance and freezing survival.

Discussion

Turions of aquatic plants are primarily adapted to overwintering in the hypoxic or anoxic conditions found in the sediments at the bottom of water courses. Here, the temperature is above freezing. Some turions, however, overwinter above the surface on wet substrates where they are subject to drying out and frost (Adamec, 1999a,b, 2008a). Therefore, on the basis of their dormant state and a low water content, a certain degree of turion frost resistance, conditioned by frost hardening, can be expected – but it has never been studied. Moreover, natural frost temperatures, to which turions overwintering above water on the wet organic topsoil can be subjected in the temperate zone, have never been monitored. In our study during the 2012–2013 winter season, the minimum air screen temperature measured near Třeboň (ca. 49° N, 15° E), as meteorological standard at 2 m above soil surface, was as low as −15.5 °C (Dušek et al., unpubl. data), while a minimum of only −4.2 °C, even without snow cover, was monitored on the wet topsoil in our outdoor experiment. For 75 days, the topsoil temperature fell below freezing and could thus induce frost hardiness in the turions, while the control turions in the experiment overwintered at above freezing temperatures in a refrigerator that simulated natural conditions under water at the bottom of a lake or a river.

The results show that dormant, non-hardened turions of 9 aquatic plant species froze between −7 and −10.2 °C (Table 1), but their survival after the measured freezing event was usually zero or very low. Moreover, no significant difference in the freezing temperatures was found for turions of *Aldrovanda*, *U. australis*, *U. bremii* and *U. ochroleuca* that were allowed to germinate or even resume sprouting for 1–6 days, being not frost resistant. In line with this finding, no difference in the levels of freezing exothermes was found for thawed *U. australis* turions or evidently dead *U. ochroleuca* turions the appearance of which had turned to a black color. Surprisingly, non-dormant, growing shoot apices of tropical *Aldrovanda* froze also at −8.5 °C which was significantly lower than in dormant temperate turions of the same species (−7.0 °C). According to the general theory (e.g., Beck, 1994; Larcher, 1995; Guy, 2003; Körner, 2003), this can altogether be interpreted that non-hardened turions are adapted to avoid freezing at temperatures of −7 to −10 °C, but after they really freeze their survival is very low (Table 1). The frost avoidance of *Hydrocharis* turions is evidently much lower and also the frost resistance of its hardened turions (only 76% germination rate) is markedly the lowest of all tested species (Table 2). On the other hand, frost-hardened turions of 7 species were able to survive outdoors, under nearly-natural winter conditions (76–100% survival rate). Although their freezing

Table 2
Survival and freezing temperatures of hardened turions of aquatic plants after overwintering outdoors in porous bags on the surface of wet topsoil.

Species	Survival (%)	DW (% FW)	Freezing temp. (°C)	Survival after measur. (%)
Aldrovanda vesiculosa	100	29.8	−2.81 ± 0.08	25
Utricularia australis	100	33.0	−3.06 ± 0.12	100
Utricularia bremii	100	31.6	−3.32 ± 0.16	100
Utricularia ochroleuca	100	28.2	−3.06 ± 0.29	100
Utricularia stygia	100	30.8	−2.88 ± 0.08	75
*Utricularia stygia**	100	30.8	−3.00 ± 0.13	100
Caldesia parnassifolia	100	37.8	−3.08 ± 0.07	0
Hydrocharis m.-ranae	75.7 ± 4.3	38.0	–	–

The asterisk denotes *U. stygia* turions that overwintered on the wet topsoil next to the porous bags; $n = 4$ bags. The values of turion survival before the measurement and after are shown in %. DW expressed in % of FW. During the measurement, 8 turions were exposed to between −9 and −10 °C. Means ± SE are shown; $n = 7$.

temperatures were only −2.8 to −3.3 °C, their survival rate after the short-term experimental freezing (exposure up to −8 to −10 °C) was very high (75–100%) – at least in all *Utricularia* species. These characteristics suggest that turions of these aquatic plants can be hardened by weak frosts and that their frost hardiness is based on a shift from frost avoidance to frost tolerance. In this respect, turions of these aquatic plants behave in the same way as winter buds of terrestrial herbs or trees (cf. Sakai and Larcher, 1987; Beck, 1994; Larcher, 1995; Guy, 2003; Körner, 2003). In frost hardened turions of all 7 species that overwintered on the wet topsoil, the water content declined by 1.7–16 percentage points due to mild drying, in spite of a substantial loss of the carbohydrate reserves over winter (Winston and Gorham, 1979a; Adamec, 1999b, 2003, 2008b).

During the measurements, the freezing of turions was indicated as a transient temperature rise (freezing exotherm) by about 2–5.6 °C. On basis that water's specific heat of fusion is 335 kJ kg^{-1} and assuming that the turions' dry biomass is relatively negligible (mean DW proportion is only ~25%), the measured temperature rise of 4.3–5.6 °C, associated with turion freezing (for *Aldrovanda*, *U. australis*, *U. ochroleuca* and *U. intermedia*), is equivalent to the freezing of only approx. 5.3–7.0% of the total water content of the turions. As the majority of the turion water content is present in vacuoles and cytoplasm, it is quite obvious that the frozen water represents the extracellular water located in cell walls (see e.g., Larcher, 1995). The very rapid dynamics of freezing this water (ca. 6–12 s) may indicate the all-or-none rule of freezing. However, on the basis of the freezing temperature recordings, no freezing of the intracellular turion water content was indicated at temperatures down to −10 to −11 °C.

It was demonstrated that different anatomical structures within an aerial winter bud might possess different sensitivities to frost (Larcher, 1995). The same may apply to turions of aquatic plants. Non-hardened, dormant turions of *U. australis*, *U. ochroleuca* and *U. stygia* were not able to sprout, and all of them died after the freezing temperature measurements. Yet, some of them exhibited weak symptoms of germination at the turion bases (the basal leaflets were partly erected) during subsequent germination tests. This suggests that the apical meristematic parts are more sensitive to frost than the basal ones.

Although turions of aquatic plants are primarily adapted to overwintering at above freezing temperatures under water, it is obvious that a certain frost resistance markedly broadens the species tolerance and ecological amplitudes. In the rooted aquatic species *Caldesia parnassifolia* zero survival was found of hardened turions after the measurements. This suggests that this species, which overwinters and sprouts at the bottom under water, is less frost tolerant than the other investigated, non-rooting species. Nevertheless, no significant differences in the frost resistance or hardiness were found in this study between the species, even if they are differing in their functional traits (rooted vs. non-rooted, sprouting at the bottom vs. at the water surface).

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